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PATENT APPLICATION

CHEMOKINE RECEPTORS AND DISEASE

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CHEMOKINE RECEPTORS AND DISEASE

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is related to USSN 60/336,849 filed October 23, 2001; USSN 60/276,797 field March 16, 2001; and USSN 60/272,330 filed February 28, 2001; each of which is incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to the identification of chemokine receptors associated with cancers and other diseases. The invention also provides for the use of agonists and antagonists of chemokine receptors in diagnosis and therapy of disease. The invention further relates to methods for identifying and using agents and/or targets that inhibit diseases associated with the chemokine receptors.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small molecular weight cytokines that induce migration and activation of leukocytes (*see*, Baggiolini M., *et al. Annu. Rev. Immunol.* 15: 675-705 (1997)). Over 30 different human chemokines have been described. They vary in their specificity for different leukocyte types (neutrophils, monocytes, eosinophils, basophils, lymphocytes, dendritic cells, *etc.*), and in the types of cells and tissues where the chemokines are synthesized. These molecules are ligands for seven transmembrane G protein linked receptors that induce a signaling cascade costimulation for T cell activation in addition to participating in transendothelial migration of leukocytes (Oppenheim *et al. Ann. Rev. Immunol.* 9:617-648 (1991), Premback *et al. Nat. Med.* 2:1174-1178 (1996)).

Over twelve different human chemokine receptors are known, including CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CXCR1, CXCR2, CXCR3, and CXCR4. The chemokines are divided between two main subfamilies, referred to as CC and CXC. CC and CXC chemokines are distinct from each other in their N terminal amino acid sequence which starts either with cysteine-cysteine or cysteine-X-cysteine where X is typically another L-amino acid. Another class of chemokine receptors is represented by

CX3CR1. These receptors have a cysteine –X3-cysteine motif. The families are also distinct in their binding pattern to their receptors. For example, the CC chemokines bind to CC receptors and not to CXC receptors and vice versa.

Binding of a chemokine to its receptor typically induces intracellular signaling responses such as a transient rise in cytosolic calcium concentration, followed by cellular biological responses such as chemotaxis. Different chemokines regulate the trafficking of distinct populations of hemopoietic cells by activating specific 7-transmembrane receptors expressed by these cells.

Some chemokine receptors also serve as coreceptors for HIV, such that they interact with HIV and with the cellular CD4 receptor to facilitate viral entry into cells. In particular, CXCR4 and CCR5 have been found to be the major coreceptors , although many other chemokine and orphan receptors have also been identified as potential coreceptors for HIV-1. Therapeutic approaches based on antagonist of these receptors have been developed, some of which are currently in clinical trials (*see*, Murakami and Yamamoto *Int J Hematol* 72:412-7 (2000)). In addition, expression of CXCR4 has been associated with osteosarcoma and pancreatic cancer. S*ee*, Paoletti *et al. Int J Oncol*; 18:11-6 (2001)) and Koshiba *et al. Clin Cancer Res.* 6:3530-5 (2000)).

Other chemokine receptor-ligand pairings have been reported, and are continuously progressing in completeness. See. e.g., Zlotnik and Yoshie "Chemokines: a new classification system and their role in immunity" *Immunity* 12:121-27 (2000).

Despite advances in the field the identification of new target molecules useful in the diagnosis and treatment of cancer and other diseases is a continuing need. The present invention addresses this and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods of detecting abnormal proliferative disease, such as cancer, in a patient. The methods comprise contacting a biological sample from the patient with a polynucleotide that selectively hybridizes to a chemokine receptor polynucleotide of the invention. Alternatively, the methods may comprise contacting a biological sample from the patient with an antibody that specifically binds to a chemokine receptor of the invention. The methods of the invention can be used to detect CXCR4 for the detection of ovarian cancer cells, bladder cancer cells, lung cancer cells, head and neck cancer cells, renal cancer cells, stomach cancer cells, uterine cancer cells, colorectal cancer cells, acute lymphoblastic leukemia cells, prostate cancer cells, pancreatic cancer cells, or

cervical cancer cells. The methods can be used to detect CCR2 for the detection of glioblastoma cancer cells. The methods can be used to detect CCR1 for the detection of glioblastoma and pancreatic cancer cells. The methods can be used to detect CCR4 for the detection of ovarian cancer cells, head and neck cancer cells, renal cancer cells, stomach cancer cells, uterine cancer cells, or colorectal cancer cells. The methods can be used to detect CCR5 for the detection of prostate cancer cells, head and neck cancer cells, renal cancer cells, stomach cancer cells, uterine cancer cells, colon cancer cells, pancreatic cancer cells, and ovarian cancer cells. The methods can be used to detect CCR7 for the detection of renal cancer cells, pancreatic cancer cells, and stomach cancer cells. The methods can be used to detect CCR8 for the detection of prostate cancer cells. The methods can be used to detect CX3CR1 for the detection of glioblastoma and pancreatic cancer cells. The methods can be used to detect CXCR6 and the biological sample is designed for the detection of lung cancer cells, bladder cancer cells, prostate cancer cells, breast cancer cells, pancreatic cancer cells, and colorectal cancer cells.

The methods may be used to monitor a patient undergoing a therapeutic regimen to treat cancer. Alternatively, the methods may be used to detect disease in patient is suspected of having cancer.

The methods of the invention can also be used to other abnormal proliferative diseases. For example, the methods can be used to detect benign tumors and/or precancerous cells such as those associated with benign prostate hyperplasia (BPH). Detection of CCR5 and CCR8 are particularly useful in these methods. Alternatively, the methods can be used to detect other cell types such as those associated with angiogenesis or rheumatoid arthritis. Detection of CXCR4 is particularly useful for these purposes.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the objects outlined above, the present invention provides methods for diagnosis and treatment of diseases such as cancer, including metastatic cancer, benign tumors, precancerous cells, as well as rheumatoid arthritis and angiogenesis. The invention is based, at least in part, on the identification of increased expression of chemokine receptors in certain cancers. In particular, CXCR4 in ovarian, bladder, colorectal, lung, head and neck, renal, stomach, uterine, acute lymphoblastic leukemia, and cervical cancers; CCR4 in head and neck, renal, stomach, uterine, colorectal, and ovarian cancers; CCR1 and/or CCR2 in glioblastoma; CCR5 in prostate cancer, head and neck cancer, renal cancer, stomach

cancer, uterine cancer, colon cancer, and ovarian cancer; CCR7 in renal and stomach cancers; CCR8 in prostate cancer; and CXCR6 in lung, bladder, prostate, breast, and colorectal cancers. Increased expression of CXCR4 has also be identified in cells associated with rheumatoid arthritis and angiogenesis. Based on this advance, the present invention provides new methods and compositions for diagnosing and treating these diseases, as well as methods for screening for compositions which modulate cancer and other diseases. The importance of chemokine receptors in cancers may include both a component of trafficking interference, where the metastatic cells are directed to particular locations by the chemokine receptors and the functions in trafficking of tumor cells themselves, and a component of companion cells which may be necessary to colocalize to the site of metastasis to increase viability or capacity to grow at a new site. Those "accessory" cells may serve to provide critical functions to allow the metastatic cells to colonize and survive in that environment in critical growth stages in the metastatic process.

The natural ligand for CXCR4 is stromal cell-derived factor (SDF1). SDF1 alpha and 1 beta are small cytokines belonging to the intercrine CXC subfamily. Human DNA sequences encoding SDF1 alpha and SDF1 beta have been isolated and characterized. The SDF1 alpha and SDF1 beta genes encode proteins of 89 and 93 amino acids, respectively (Shirozu *et al.*, *Genomics* 28:495-500 (1995), *see, also* Genbank accession numbers L36033 and L36034). Thus, sequences from these proteins can also be used to modulate CXCR4 activity in cells.

The natural ligands for CCR8, which is also called Ter-1 (UniGene hs.113222, and GenBank accessions D49919, U45983, U62556, Z79782, Y08456, and NM_005201), include I-309 (UniGene hs.72918, and GenBank accessions NM_002981, NP_002972, M57506) and CCL1 (GenBank accessions M57506 and M57502).

The natural ligands for CXCR6, which is also called STRL33 and Bonzo (UniGene hs. 34526 and GenBank accessions U73531, U73529, AF007545) include CXCL16 (GenBank accession NM 022059).

The natural ligands for CCR5 (UniGene hs.54443 and GenBank accessions AF031237, XM0303397, NM 000579) include RANTES, CCL5 (AF043341); MIP-1alpha, LD78alpha, CCL3 (M23452), and MIP-1beta, CCL4 (M23502).

The natural ligands for CCR4 (UniGene hs.184926, and GenBank accessions X85740, NM_005508, AB023888, AB023889, AB023890,AB023891, AB023892)are MDC (UniGene hs.97203, and GenBank accessions NM_002990, NP_002981) and TARC (UniGene hs.66742, and GenBank accessions D43767, NM_002987, NP_002978).

The natural ligands for CCR1 (UG hs.301921 and GenBank accessions L10918, D10925, L09230, and NM_001295) are RANTES, MIP-1a, MCP-1 (UniGene hs.303649, and GenBank accessions NM_002982, NP_002973).

The natural ligands for CCR2 (UniGene hs.302043, and GenBank accessions AF014958, U97123, AF015524, AJ344142, AF015525, NM_003965 and NM 000647) are MCP-1, MCP-3 (UniGene hs.251526, and GenBank accessions NM_006277, NP_006264, X72309) and MCP-4 (UniGene hs.11383, and GenBank accessions NM_005408, NP_005399, U59808

The natural ligand for CCR7 (UniGene hs.1652, GenBank accessions L08176, L31581, NM_001838 and XM 049959)is ELC, the EBI1 ligand.

The natural ligand for CX3CR1 (UniGene hs.78913, and GenBank accessions U20350, U28934, NM_001337 XM_047502) is fractalkine (UniGene hs.80420, and GenBank accessions NM_002996, NP_2987).

The natural ligands for CXCR3 (UniGene hs.198252, and GenBank accessions NM_001504, NP_001495, U32674, Z79783) are I-TAC (UniGene hs.103982, and GenBank accessions NM_005409, NP_005400, AF30514, U66096, Y15220), IP-10 UniGene hs.2248, and GenBank accessions NM_001565, NP_001556, X02530) and MIG (UniGene hs.77367, and GenBank accessions NM_002416, NP_002407, X72755).

All UniGene cluster identification numbers (see, http://www.ncbi.nlm.nih.gov/unigene/).and accession numbers herein are for the GenBank sequence database and the sequences of the accession numbers are hereby expressly incorporated by reference. GenBank is known in the art, *see, e.g.,* Benson, DA, *et al.*, Nucleic Acids Research 26:1-7 (1998) and http://www.ncbi.nlm.nih.gov/. Sequences are also available in other databases, e.g., European Molecular Biology Laboratory (EMBL) and DNA Database of Japan (DDBJ).

Table 1 presents a listing of the chemokine receptor, the indications correlated with expression of that receptor, and natural ligands of the receptors. See Zlotnik and Yoshie *Immunity* 12:121-27 (2000).

Table 1:

CXCR4	Ovarian, bladder, colorectal, lung, head & neck, renal, stomach, uterine, acute lymphoblastic leukemia, cervical, glioblastoma, pancreatic and prostate cancers; also BPH, angiogenesis and rheumatoid arthritis	SDF1
CCR1	Glioblastoma and pancreatic cancer	RANTES, MIP-1a, MCP-1
CCR2	Glioblastoma and angiogenesis	MCP-1, MCP-3, MCP-4
CCR4	head & neck, renal, stomach, uterine, colorectal, glioblastoma and ovarian cancers	MDC, TARC
CCR5	Prostate, head and neck, renal, stomach, uterine, colorectal, pancreatic and ovarian cancers BPH	RANTES (CCL5), MIP-1a (LD78a, CCL3), MIP-1b (CCL4)
CCR7	renal, pancreatic and stomach cancers	6Ckine (SLC)
CCR8 (Ter-1)	glioblastoma, and prostate cancer; BPH	I-309 (CCL1)
CX3CR1	glioblastoma and pancreatic cancer	Fractalkine (CX3CL1)
CXCR3	Glioblastoma	I-TAC, IP-10 and MIG
CXCR6 (STRL33)	lung, bladder, prostate, breast, pancreatic and colorectal cancers and BPH	CXCL16

Definitions

The terms "CXCR4 polynucleotide" and "CXCR4 polypeptide" refer to nucleic acid and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have a nucleotide sequence that has greater than about 60% nucleotide sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater nucleotide sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more nucleotides, to Unigene hs.89414, and GenBank Accessions No. AF025375 L08176, L31581, NM_001838; (2) bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence as shown in these accessions (3) specifically hybridize under stringent hybridization conditions to a nucleic acid sequence, or the complement thereof of the sequences in these accessions; or (4) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 350, or more amino acids, to the amino acid sequences noted above. A polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or another mammal. A "CXCR4 polypeptide" and a "CXCR4 polynucleotide," may be either naturally occurring or recombinant. Similarly the terminology is used with respect to the other receptors and their sequences, e.g., CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CX3CR1 and CXCR6. See, e.g., GenBank accessions noted above.

A "full length" chemokine receptor protein or nucleic acid refers to a receptor polypeptide or polynucleotide sequence, or a variant thereof, that contains all of the elements normally contained in one or more naturally occurring, wild type receptor polynucleotide or polypeptide sequences. The "full length" may be prior to, or after, various stages of post-translation processing or splicing, including alternative splicing.

"Biological sample" as used herein is a sample of biological tissue or fluid that contains nucleic acids or polypeptides, e.g., of a chemokine receptor protein, polynucleotide or transcript. Such samples include, but are not limited to, tissue isolated from primates, e.g., humans, or rodents, e.g., mice, and rats. Biological samples may also include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes such as blood, plasma, serum, sputum, stool, tears, mucus, hair, skin, and the like. Biological samples also include explants and primary and/or transformed cell cultures derived from

patient tissues. A biological sample is typically obtained from a eukaryotic organism, most preferably a mammal such as a primate *e.g.*, chimpanzee or human; cow; dog; cat; a rodent, e.g., guinea pig, rat, mouse; rabbit; or a bird; reptile; or fish. In the present invention, the biological sample will typically be an ovarian, lung, colorectal, ovarian, head and neck, renal, stomach, uterine, blood cells, brain, prostate, or breast tissue sample, but other body fluids or samples might also be diagnostic of cancer in one of those tissues.

A biological sample is "designed for the detection of" a particular cancer cell if the sample is taken from the target organ (*e.g.*, a lung sample for detecting a lung cancer cell). In addition, the sample may be taken from an organ or tissue suspected of comprising a metastatic cell from the primary tumor. Such secondary sites may include lymph nodes or other tissues suspected of comprising metastases. Those of skill will recognize the secondary sites most commonly associated with metastasis for any particular cancer.

"Providing a biological sample" means to obtain a biological sample for use in methods described in this invention. Most often, this will be done by removing a sample of cells from an animal, but can also be accomplished by using previously isolated cells (*e.g.*, isolated by another person, at another time, and/or for another purpose), or by performing the methods of the invention *in vivo*. Archival tissues, having treatment or outcome history, will be particularly useful.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (*see, e.g.,* NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ or the like). Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as

far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequences.

A "host cell" is a naturally occurring cell or a transformed cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be cultured cells, explants, cells *in vivo*, and the like. Host cells may be

prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHO, HeLa, and the like (*see, e.g.,* the American Type Culture Collection catalog or web site, www.atcc.org).

The terms "isolated," "purified," or "biologically pure" refer to material that is substantially or essentially free from components that normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein or nucleic acid that is the predominant species present in a preparation is substantially purified. In particular, an isolated nucleic acid is separated from open reading frames that flank the gene and encode proteins other than protein encoded by the gene. The term "purified" in some embodiments denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Preferably, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure. "Purify" or "purification" in other embodiments means removing at least one contaminant from the composition to be purified. In this sense, purification does not require that the purified compound be 100% pure.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan

(W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g.,* Creighton, *Proteins* (1984)).

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, *see, e.g.,* Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor & Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 25 to approximately 500 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β-sheet and α-helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed, usually by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

"Nucleic acid" or "oligonucleotide" or "polynucleotide" or grammatical equivalents used herein means at least two nucleotides covalently linked together. Oligonucleotides are typically from about 5, 6, 7, 8, 9, 10, 12, 15, 15, 25, 30, 40, 50 or more nucleotides in length, up to about 100 nucleotides in length. Nucleic acids and polynucleotides are a polymer of any length. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press); and peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with positive backbones; non-ionic backbones, and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, *Carbohydrate Modifications in Antisense Research*, Sanghui & Cook, eds.. Nucleic acids containing one or more carbocyclic sugars are also included within one definition of nucleic acids. Modifications of the ribose-phosphate backbone may be done for a variety of reasons, for example to increase the stability and half-life of such molecules in physiological environments or as probes on a biochip. Mixtures of naturally occurring

nucleic acids and analogs can be made; alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (T_m) for mismatched versus perfectly matched base pairs. DNA and RNA typically exhibit a 2-4°C drop in T_m for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. In addition, PNAs are not degraded by cellular enzymes, and thus can be more stable.

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand also defines the sequence of the complementary strand; thus the sequences described herein also provide the complement of the sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc. "Transcript" typically refers to a naturally occurring RNA, e.g., a pre-mRNA, hnRNA, or mRNA. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

An "effector" or "effector moiety" or "effector component" is a molecule that is bound (or linked, or conjugated), either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds, to an antibody.

The "effector" can be a variety of molecules including, for example, detection moieties including radioactive compounds, fluorescent compounds, an enzyme or substrate, tags such as epitope tags, a toxin; a chemotherapeutic agent; a lipase; an antibiotic; or a radioisotope emitting "hard" *e.g.*, beta radiation.

A "labeled nucleic acid probe or oligonucleotide" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe. Alternatively, method using high affinity interactions may achieve the same results where one of a pair of binding partners binds to the other, *e.g.*, biotin, streptavidin.

As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid, e.g., using polymerases and endonucleases, in a form not normally found in nature. Thus an isolated nucleic acid, in a linear form, or an expression vector formed *in*

vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e., using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention. Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid as depicted above.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42° C, or, 5x SSC, 1% SDS, incubating at 65° C, with wash in 0.2x SSC, and 0.1% SDS at 65° C. For PCR, a temperature of about 36° C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification

reactions are provided, e.g., in Innis *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37° C, and a wash in 1X SSC at 45° C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, *e.g.*, and Current Protocols in Molecular Biology, ed. Ausubel, *et al*

"Inhibitors", "activators", and "modulators" of, e.g., CXCR4 polynucleotide and polypeptide sequences are used to refer to activating, inhibitory, or modulating molecules or compounds identified using *in vitro* and *in vivo* assays of CXCR4 polynucleotide and polypeptide sequences. Inhibitors are compounds that, e.g., bind to, partially or totally block activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity or expression of CXCR4 proteins, e.g., antagonists. "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate CXCR4 protein activity. Inhibitors, activators, or modulators also include genetically modified versions of the proteins, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, ligand homologs, antibodies, small chemical molecules and the like. Such assays for inhibitors and activators include, e.g., expressing the CXCR4 protein *in vitro*, in cells, or cell membranes, applying putative modulator compounds, and then determining the functional effects on activity, as described above. Activators and inhibitors of CXCR4 can also be identified by incubating cancer cells with the test compound and determining increases or decreases in the expression of 1 or more cancer proteins, *e.g.*, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 or more cancer proteins.

Samples or assays comprising chemokine receptor proteins that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative protein activity value of 100%. Inhibition of a polypeptide is achieved when the activity value relative to the control is about 80%,

preferably 50%, more preferably 25-0%. Activation of a receptor polypeptide is achieved when the activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

The phrase "changes in cell growth" refers to any change in cell growth and proliferation characteristics *in vitro* or *in vivo*, such as formation of foci, anchorage independence, semi-solid or soft agar growth, changes in contact inhibition and density limitation of growth, loss of growth factor or serum requirements, changes in cell morphology, gaining or losing immortalization, gaining or losing tumor specific markers, ability to form or suppress tumors when injected into suitable animal hosts, and/or immortalization of the cell. *See, e.g.,* Freshney, *Culture of Animal Cells a Manual of Basic Technique* pp. 231-241 (3rd ed. 1994).

"Tumor cell" refers to precancerous, cancerous, and normal cells in a tumor.

"Cancer cells," "transformed" cells or "transformation" in tissue culture, refers to spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic DNA, or uptake of exogenous DNA, it can also arise spontaneously or following exposure to a carcinogen, thereby mutating an endogenous gene. Transformation is associated with phenotypic changes, such as immortalization of cells, aberrant growth control, and/or malignancy (*see*, Freshney, *Culture of Animal Cells a Manual of Basic Technique* (3rd ed. 1994)).

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily

responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'$_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (*see, e.g.,* McCafferty *et al.*, *Nature* 348:552-554 (1990))

For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (*see, e.g.,* Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4:72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy* (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.,* McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable

region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

Identification of receptor and ligand sequences

The following discussion focuses on use of CXCR4 genes and proteins, as well as the corresponding ligands, in the diagnosis and treatment of diseases. One of skill will recognize that this discussion applies to the other chemokine receptors and their ligands disclosed here (*e.g.*, , CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CX3CR1 and CXCR6). In one aspect, the expression levels of, e.g., CXCR4 genes and other genes are determined in different patient samples for which diagnosis information is desired, to provide expression profiles. An expression profile of a particular sample is essentially a "fingerprint" of the state of the sample; while two states may have any particular gene similarly expressed, the evaluation of a number of genes simultaneously allows the generation of a gene expression profile that is unique to the state of the cell. That is, normal tissue may be distinguished from cancerous or metastatic cancerous tissue, or metastatic cancerous tissue can be compared with tissue from surviving cancer patients. By comparing expression profiles of tissue in known different cancer states, information regarding which genes are important (including both up- and down-regulation of genes) in each of these states is obtained.

The identification of CXCR4 sequences and other sequences in cancer versus non-cancer tissue allows the use of this information in a number of ways. For example, a particular treatment regime may be evaluated: does a chemotherapeutic drug act to down-regulate cancer, and thus tumor growth or recurrence, in a particular patient. Similarly, diagnosis and treatment outcomes may be done or confirmed by comparing patient samples with the known expression profiles. Metastatic tissue can also be analyzed to determine the stage of cancer in the tissue. Furthermore, these gene expression profiles (or individual genes) allow screening of drug candidates with an eye to mimicking or altering a particular expression profile; for example, screening can be done for drugs that suppress the cancer expression profile. This may be done by making biochips comprising sets of the important cancer genes, which can then be used in these screens. These methods can also be done on the protein basis; that is, protein expression levels of CXCR4 can be evaluated for diagnostic purposes or to screen candidate agents. In addition, the CXCR4 nucleic acid sequences can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or the CXCR4 proteins (including antibodies and other modulators thereof) administered as therapeutic drugs.

Use of receptor and ligand nucleic acids

Nucleic acids encoding the receptors and ligands of the invention are used in several ways. For example, nucleic acid probes comprising CXCR4 sequences are made and attached to biochips (along with other cancer-specific nucleic acids) to be used in screening and diagnostic methods to detect disease, as outlined below. Alternatively, the CXCR4 and SDF1 nucleic acids can be used for administration, for example for gene therapy, vaccine, and/or antisense applications. Alternatively, the CXCR4 and SDF1 nucleic acids that include coding regions of CXCR4 or SDF1 polypeptides can be put into expression vectors for the expression of polypeptides, again for screening purposes or for administration to a patient.

In a preferred embodiment, nucleic acid probes that specifically hybridize to receptor or ligand nucleic acids, e.g., CXCR4 or SDF1, are made. The CXCR4 nucleic acid probes attached to the biochip are designed to be substantially complementary to CXCR4 nucleic acids, *i.e.* the target sequence (either the target sequence of the sample or to other probe sequences, for example, in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be a number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.

A nucleic acid probe is generally single stranded but can be partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. In general, the nucleic acid probes range from about 8 to about 100 bases long, with from about 10 to about 80 bases being preferred, and from about 30 to about 50 bases being particularly preferred. That is, generally whole genes are not used. In some embodiments, much longer nucleic acids can be used, up to hundreds of bases.

In a preferred embodiment, more than one probe per sequence is used, with either overlapping probes or probes to different sections of the target being used. That is, two, three, four or more probes, with three being preferred, are used to build in a redundancy

for a particular target. The probes can be overlapping (i.e., have some sequence in common), or separate. In some cases, PCR primers may be used to amplify signal for higher sensitivity.

As will be appreciated by those in the art, nucleic acids can be attached or immobilized to a solid support in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can typically be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one or more of electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

The biochip comprises a suitable solid substrate. By "substrate" or "solid support" or other grammatical equivalents herein is meant a material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TeflonJ, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not appreciably fluoresce. A preferred substrate is described in an application entitled Reusable Low Fluorescent Plastic Biochip, U.S. Application Serial No. 09/270,214, filed March 15, 1999, herein incorporated by reference in its entirety.

Generally the substrate is planar, although as will be appreciated by those in the art, other configurations of substrates may be used as well. For example, the probes may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Similarly, the substrate may be flexible, such as a flexible foam, including closed cell foams made of particular plastics.

In a preferred embodiment, the surface of the biochip and the probe may be derivatized with chemical functional groups for subsequent attachment of the two. Thus, for example, the biochip is derivatized with a chemical functional group including, but not limited to, amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. Using these functional groups, the probes can be attached using functional groups on the probes. For example, nucleic acids containing amino groups can be attached to surfaces comprising amino groups, for example using linkers as are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (*see* 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200). In addition, in some cases, additional linkers, such as alkyl groups (including substituted and heteroalkyl groups) may be used.

In this embodiment, oligonucleotides are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside.

In another embodiment, the immobilization to the solid support may be very strong, yet non-covalent. For example, biotinylated oligonucleotides can be made, which bind to surfaces covalently coated with streptavidin, resulting in attachment.

Alternatively, the oligonucleotides may be synthesized on the surface, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques are used. In a preferred embodiment, the nucleic acids can be synthesized in situ, using well known photolithographic techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Patent Nos. 5,700,637 and 5,445,934; and references cited within, all of which are expressly incorporated by reference; these methods of attachment form the basis of the Affymetrix GeneChip™ technology.

Often, amplification-based assays are performed to measure the expression level of receptor or ligand, e.g., CXCR4 and SDF1 sequences. These assays are typically performed in conjunction with reverse transcription. In such assays, a nucleic acid sequence acts as a template in an amplification reaction (e.g., Polymerase Chain Reaction, or PCR). In

a quantitative amplification, the amount of amplification product will be proportional to the amount of template in the original sample. Comparison to appropriate controls provides a measure of the amount of CXCR4 RNA. Methods of quantitative amplification are well known to those of skill in the art. Detailed protocols for quantitative PCR are provided, e.g., in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications* (1990).

In some embodiments, a TaqMan based assay is used to measure expression. TaqMan based assays use a fluorogenic oligonucleotide probe that contains a 5' fluorescent dye and a 3' quenching agent. The probe hybridizes to a PCR product, but cannot itself be extended due to a blocking agent at the 3' end. When the PCR product is amplified in subsequent cycles, the 5' nuclease activity of the polymerase, *e.g.*, AmpliTaq, results in the cleavage of the TaqMan probe. This cleavage separates the 5' fluorescent dye and the 3' quenching agent, thereby resulting in an increase in fluorescence as a function of amplification (*see*, for example, literature provided by Perkin-Elmer, *e.g.*, www2.perkin-elmer.com).Other suitable amplification methods include, but are not limited to, ligase chain reaction (LCR) (*see* Wu & Wallace, *Genomics* 4:560 (1989), Landegren *et al.*, *Science* 241:1077 (1988), and Barringer *et al.*, *Gene* 89:117 (1990)), transcription amplification (Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1173 (1989)), self-sustained sequence replication (Guatelli *et al.*, *Proc. Nat. Acad. Sci. USA* 87:1874 (1990)), dot PCR, and linker adapter PCR, etc.

Expression of receptor or ligand polypeptides from nucleic acids

In a preferred embodiment, receptor or ligand, e.g., CXCR4 and/or SDF1, nucleic acids are used to make a variety of expression vectors to express polypeptides which can then be used in screening assays, as described below. Expression vectors and recombinant DNA technology are well known to those of skill in the art (*see, e.g.,* Ausubel, *supra*, and *Gene Expression Systems* (Fernandez & Hoeffler, eds, 1999)) and are used to express proteins. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the CXCR4 protein. The term "control sequences" refers to DNA sequences used for the expression of an operably linked coding sequence in a particular host organism. Control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is typically accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. Transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the protein. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

In general, transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

In addition, an expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art (*e.g.*, Fernandez & Hoeffler, *supra*).

In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

The receptor or ligand, e.g., CXCR4 and SDF1, polypeptides of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a protein, under the appropriate conditions to induce or cause expression of the protein. Conditions appropriate for protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation or optimization. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculovirus systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

Appropriate host cells include yeast, bacteria, archaebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are *Saccharomyces cerevisiae* and other yeasts, *E. coli, Bacillus subtilis*, Sf9 cells, C129 cells, 293 cells, *Neurospora*, BHK, CHO, COS, HeLa cells, HUVEC (human umbilical vein endothelial cells), THP1 cells (a macrophage cell line) and various other human cells and cell lines.

In a preferred embodiment, the receptor or ligand proteins, e.g., CXCR4 and SDF1, are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral and adenoviral systems. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter (*see, e.g.,* Fernandez & Hoeffler, *supra*). Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. Examples of transcription terminator and polyadenylation signals include those derived form SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection,

encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

In a preferred embodiment, CXCR4 or SDF1 polypeptides are expressed in bacterial systems. Bacterial expression systems are well known in the art. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the tac promoter is a hybrid of the trp and lac promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. The expression vector may also include a signal peptide sequence that provides for secretion of the CXCR4 protein in bacteria. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways. These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis, E. coli, Streptococcus cremoris*, and *Streptococcus lividans*, among others (e.g., Fernandez & Hoeffler, *supra*). The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

In one embodiment, proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art.

In a preferred embodiment, polypeptides are produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae, Candida albicans* and *C. maltosa, Hansenula polymorpha, Kluyveromyces fragilis* and *K. lactis, Pichia guillerimondii and P. pastoris, Schizosaccharomyces pombe*, and *Yarrowia lipolytica*.

The receptor or ligand polypeptides may also be made as fusion proteins, using techniques well known in the art. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the polypeptide may be fused to a carrier protein to

form an immunogen. Alternatively, the protein may be made as a fusion protein to increase expression, or for other reasons.

In a preferred embodiment, the receptor or ligand, e.g., CXCR4 or SDF1, polypeptide is purified or isolated after expression. The polypeptides may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the protein may be purified using a standard anti-CXCR4 or anti-SDF1 protein antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see Scopes, *Protein Purification* (1982). The degree of purification necessary will vary depending on the use of the protein. In some instances no purification will be necessary.

Once expressed and purified if necessary, the proteins and nucleic acids are useful in a number of applications. They may be used as immunoselection reagents, as vaccine reagents, as screening agents, etc.

Variants of receptor, e.g., CXCR4, polypeptides

In one embodiment, the receptor or ligand, e.g., CXCR4 or SDF1, polypeptides are derivative or variant proteins as compared to the wild-type sequence. That is, as outlined more fully below, the derivative peptide will often contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the peptide.

Also included within one embodiment of polypeptides of the present invention are amino acid sequence variants. These variants typically fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant protein fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the protein amino acid

sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed receptor, e.g., CXCR4, variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of, e.g., CXCR4 protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or combinations thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the receptor protein, e.g., CXCR4, are desired, substitutions are generally made in accordance with the amino acid substitution chart provided in the definition section.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analog, although variants also are selected to modify the characteristics of the receptor proteins as needed. Alternatively, the variant may be designed such that the biological activity of the receptor protein is altered. For example, glycosylation sites may be altered or removed.

Covalent modifications of receptor polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a receptor polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of a receptor polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking receptor polypeptides to a water-insoluble support matrix or surface for use in the method for purifying anti-receptor polypeptide antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-

phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-((p-azidophenyl)dithio)propioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the γ-amino groups of lysine, arginine, and histidine side chains (Creighton, *Proteins: Structure and Molecular Properties*, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of a receptor polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence receptor polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence receptor polypeptide. Glycosylation patterns can be altered in many ways. For example the use of different cell types to express receptor sequences can result in different glycosylation patterns.

Addition of glycosylation sites to receptor polypeptides may also be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence receptor polypeptide (for O-linked glycosylation sites). The receptor amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the receptor polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the receptor polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330, and in Aplin & Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of carbohydrate moieties present on the receptor polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, *et al.*, *Arch.*

Biochem. Biophys., 259:52 (1987) and by Edge *et al.*, *Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.*, 138:350 (1987).

Another type of covalent modification of receptor comprises linking the receptor polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Receptor polypeptides may also be modified in a way to form chimeric molecules comprising a receptor polypeptide fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of, e.g., a CXCR4 polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the CXCR4 polypeptide. The presence of such epitope-tagged forms of a CXCR4 polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the CXCR4 polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of a CXCR4 polypeptide with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; His6 and metal chelation tags, the flu HA tag polypeptide and its antibody 12CA5 (Field *et al.*, *Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, *Molecular and Cellular Biology* 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (*Paborsky et al.*, *Protein Engineering* 3(6):547-553 (1990)). Other tag polypeptides include the Flag-peptide (*Hopp et al.*, *BioTechnology* 6:1204-1210 (1988)); the KT3 epitope peptide (Martin *et al.*, *Science* 255:192-194 (1992)); tubulin epitope peptide (Skinner *et al.*, *J. Biol. Chem.* 266:15163-15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6393-6397 (1990)).

Also included with an embodiment of CXCR4 protein are other chemokine receptor proteins, and chemokine receptor proteins from other organisms, which are cloned

and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related proteins from humans or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the unique areas of the CXCR4 nucleic acid sequence. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain inosine as needed. The conditions for the PCR reaction are well known in the art (e.g., Innis, PCR Protocols, *supra*).

Antibodies to receptor or ligand proteins

In a preferred embodiment, when the receptor or ligand protein is to be used to generate antibodies, e.g., for immunotherapy or immunodiagnosis, the receptor or ligand protein should share at least one epitope or determinant with the full length protein. By "epitope" or "determinant" herein is typically meant a portion of a protein which will generate and/or bind an antibody or T-cell receptor in the context of MHC. Thus, in most instances, antibodies made to a smaller protein will be able to bind to the full-length protein, particularly linear epitopes. In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity.

Methods of preparing polyclonal antibodies are known to the skilled artisan (e.g., Coligan, *supra*; and Harlow & Lane, *supra*). Polyclonal antibodies can be raised in a mammal, *e.g.*, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include a protein encoded by a nucleic acid of the figures or fragment thereof or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

The antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler & Milstein, *Nature* 256:495 (1975). In a hybridoma method, a mouse, hamster, or other

appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro. The immunizing agent will typically include a polypeptide encoded by a nucleic acid of the invention or fragment thereof, or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (1986)). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

In one embodiment, the antibodies are bispecific antibodies. Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens or that have binding specificities for two epitopes on the same antigen. In one embodiment, one of the binding specificities is for CXCR4, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit, preferably one that is tumor specific. Alternatively, tetramer-type technology may create multivalent reagents.

In a preferred embodiment, the antibodies to, e.g., CXCR4 or SDF1 proteins are capable of reducing or eliminating a biological function of a CXCR4 protein, as is described below. That is, the addition of anti-CXCR4 protein antibodies (either polyclonal or preferably monoclonal) to CXCR4 expressing tissue (or cells containing CXCR4) may reduce or eliminate the cancer associated with expression of the protein. Generally, at least a 25% decrease in activity is preferred, with at least about 50% being particularly preferred and about a 95-100% decrease being especially preferred.

In a preferred embodiment the antibodies to the CXCR4 or SDF1 proteins are humanized antibodies (e.g., Xenerex Biosciences, Medarex, Inc., Abgenix, Inc., Protein Design Labs, Inc.) Humanized forms of non-human (e.g., murine) antibodies are chimeric

molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework (FR) regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992)).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-327 (1988); Verhoeyen *et al.*, *Science* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom & Winter, *J. Mol. Biol.* 227:381 (1991); Marks *et al.*, *J. Mol. Biol.* 222:581 (1991)). The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*,

Monoclonal Antibodies and Cancer Therapy, p. 77 (1985) and Boerner *et al., J. Immunol.* 147(1):86-95 (1991)). Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, *Bio/Technology* 10:779-783 (1992); Lonberg *et al., Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild *et al., Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); Lonberg & Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995).

By immunotherapy is meant treatment of cancer (ovarian, lung, colorectal, bladder, head and neck, renal, stomach, uterine, glioblastoma, prostate, or breast cancer) with an antibody raised against the appropriate receptor or ligand, e.g., CXCR4 or SDF1, proteins. As used herein, immunotherapy can be passive or active. Passive immunotherapy as defined herein is the passive transfer of antibody to a recipient (patient). Active immunization is the induction of antibody and/or T-cell responses in a recipient (patient). Induction of an immune response is the result of providing the recipient with an antigen to which antibodies are raised. As appreciated by one of ordinary skill in the art, the antigen may be provided by injecting a polypeptide against which antibodies are desired to be raised into a recipient, or contacting the recipient with a nucleic acid capable of expressing the antigen and under conditions for expression of the antigen, leading to an immune response.

Without being bound by theory, anti-receptor antibodies used for treatment, bind the extracellular domain of the receptor protein and prevent it from binding to other proteins, such as circulating chemokine ligand. The antibody may cause down-regulation of the transmembrane receptor protein. As will be appreciated by one of ordinary skill in the art, the antibody may be a competitive, non-competitive or uncompetitive inhibitor of protein binding to the extracellular domain of the receptor protein. The antibody is also an antagonist of the receptor protein. Further, the antibody prevents activation of the transmembrane receptor. In one aspect, when the antibody prevents the binding of other molecules to the receptor protein, the antibody prevents receptor function, e.g., activation or signaling.

The antibody may also be used to target or sensitize the cell to cytotoxic agents, including, but not limited to TNF-α, TNF-β, IL-1, INF-γ and IL-2, or chemotherapeutic agents including 5FU, vinblastine, actinomycin D, cisplatin, methotrexate, and the like. In some instances the antibody belongs to a sub-type that activates serum complement when complexed with the transmembrane protein thereby mediating cytotoxicity or antigen-dependent cytotoxicity (ADCC). Thus, receptor is treated by administering to a patient antibodies directed against the transmembrane receptor protein. Antibody-labeling may activate a co-toxin, localize a toxin payload, or otherwise provide means to locally ablate cells.

In another preferred embodiment, the antibody is conjugated to an effector moiety. The effector moiety can be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the therapeutic moiety is a small molecule that modulates the activity of the receptor protein.

In a preferred embodiment, the therapeutic moiety can also be a cytotoxic agent. In this method, targeting the cytotoxic agent to receptor, e.g., CXCR4 tissue or cells, results in a reduction in the number of afflicted cells, thereby reducing symptoms associated with CXCR4. Cytotoxic agents are numerous and varied and include, but are not limited to, cytotoxic drugs or toxins or active fragments of such toxins. Suitable toxins and their corresponding fragments include diphtheria A chain, exotoxin A chain, ricin A chain, abrin A chain, curcin, crotin, phenomycin, enomycin, saporin, and the like. Cytotoxic agents also include radiochemicals made by conjugating radioisotopes to antibodies raised against receptor proteins, or binding of a radionuclide to a chelating agent that has been covalently attached to the antibody. Targeting the therapeutic moiety to transmembrane receptor proteins not only serves to increase the local concentration of therapeutic moiety in the receptor afflicted area, but also serves to reduce deleterious side effects that may be associated with the therapeutic moiety.

The CXCR4 or SDF1 antibodies of the invention specifically bind to CXCR4 or SDF1 proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a K_d of at least about 0.1 mM, more usually at least about 1 μM, preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better. Selectivity of binding is also important. The examples of CXCR4 and SDF1 are exemplary of the receptor and ligand matching and correlation to specific cancers as described herein.

Detection of receptor sequence for diagnostic and therapeutic applications

In one aspect, the expression levels of receptor, e.g., CXCR4 genes are determined for detecting cancer cells. Evaluation may be at the gene transcript, or the protein level. The amount of gene expression may be monitored using nucleic acid probes to the DNA or RNA equivalent of the gene transcript, and the quantification of gene expression levels, or, alternatively, the final gene product itself (protein) can be monitored, e.g., with antibodies to the CXCR4 protein and standard immunoassays (ELISAs, etc.) or other techniques, including mass spectroscopy assays, 2D gel electrophoresis assays, etc. Proteins corresponding to CXCR4 genes, i.e., those identified as being important in a cancer phenotype, can be evaluated in a diagnostic test.

In a preferred embodiment, gene expression monitoring is performed simultaneously on a number of genes. Multiple protein expression monitoring can be performed as well. Similarly, these assays may be performed on an individual basis as well.

In one embodiment, the CXCR4 nucleic acid probes are attached to biochips as outlined herein for the detection and quantification of CXCR4 sequences in a particular cell. PCR techniques can be used to provide greater sensitivity. Similarly, other receptors can be detected.

In a preferred embodiment nucleic acids encoding the receptor protein, e.g., CXCR4, are detected. Although DNA or RNA encoding the CXCR4 protein may be detected, of particular interest are methods wherein an mRNA encoding a CXCR4 protein is detected. Probes to detect mRNA can be a nucleotide/deoxynucleotide probe that is complementary to and hybridizes with the mRNA and includes, but is not limited to, oligonucleotides, cDNA or RNA. Probes also should contain a detectable label, as defined herein. In one method the mRNA is detected after immobilizing the nucleic acid to be examined on a solid support such as nylon membranes and hybridizing the probe with the sample. Following washing to remove the non-specifically bound probe, the label is detected. In another method detection of the mRNA is performed in situ. In this method permeabilized cells or tissue samples are contacted with a detectably labeled nucleic acid probe for sufficient time to allow the probe to hybridize with the target mRNA. Following washing to remove the non-specifically bound probe, the label is detected. For example a digoxygenin labeled riboprobe (RNA probe) that is complementary to the mRNA encoding a CXCR4 protein is detected by binding the digoxygenin with an anti-digoxygenin secondary antibody and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate.

In another preferred method, antibodies to the receptor protein, e.g., CXCR4, find use in *in situ* imaging techniques, *e.g.*, in histology (*e.g.*, *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993)). In this method cells are contacted with from one to many antibodies to the CXCR4 protein. Following washing to remove non-specific antibody binding, the presence of the antibody or antibodies is detected. In one embodiment the antibody is detected by incubating with a secondary antibody that contains a detectable label. In another method the primary antibody to the CXCR4 protein contains a detectable label, for example an enzyme marker that can act on a substrate. In another preferred embodiment each one of multiple primary antibodies contains a distinct and detectable label. This method finds particular use in simultaneous screening for a plurality of CXCR4 proteins. As will be appreciated by one of ordinary skill in the art, many other histological imaging techniques are also provided by the invention.

In a preferred embodiment the label is detected in a fluorometer which has the ability to detect and distinguish emissions of different wavelengths. In addition, a fluorescence activated cell sorter (FACS) can be used in the method.

In another preferred embodiment, antibodies find use in diagnosing the presence of cancer cells from blood, serum, plasma, stool, and other samples. Such samples, therefore, are useful as samples to be probed or tested for the presence of receptor proteins, e.g., CXCR4. Antibodies can be used to detect a CXCR4 protein by previously described immunoassay techniques including ELISA, immunoblotting (western blotting), immunoprecipitation, BIACORE technology and the like. Conversely, the presence of antibodies may indicate an immune response against an endogenous CXCR4 protein.

In a preferred embodiment, *in situ* hybridization of labeled receptor nucleic acid probes to tissue arrays is done. For example, arrays of tissue samples, including metastatic cancer tissue and/or normal tissue, are made. *In situ* hybridization (*see, e.g.*, Ausubel, *supra*) is then performed. When comparing the fingerprints between an individual and a standard, the skilled artisan can make a diagnosis, a prognosis, or a prediction based on the findings. It is further understood that the genes which indicate the diagnosis may differ from those which indicate the prognosis and molecular profiling of the condition of the cells may lead to distinctions between responsive or refractory conditions or may be predictive of outcomes.

In a preferred embodiment, the receptor proteins, antibodies, nucleic acids, modified proteins and cells containing receptor sequences are used in prognosis assays. As above, gene expression profiles can be generated that correlate to cancer, in terms of long

term prognosis. Again, this may be done on either a protein or gene level, with the use of genes being preferred. As above, receptor probes may be attached to biochips for the detection and quantification of receptor sequences in a tissue or patient. The assays proceed as outlined above for diagnosis. PCR method may provide more sensitive and accurate quantification.

Assays for therapeutic compounds

In a preferred embodiment a designated receptor or its ligand are used in drug screening assays to identify compounds that have a functional effect on receptor activity or expression. . The phrase "functional effects" in the context of assays for testing compounds that modulate activity of a receptor protein includes the determination of a parameter that is indirectly or directly under the influence of the protein or nucleic acid, e.g., a functional, physical, or chemical effect, such as the ability to decrease cancer or metastasis. As noted above chemokine receptor proteins are G protein coupled receptors. Assays for activation of G protein coupled receptors are well known in the art. Such assays include those that measure ligand binding (e.g., by radioactive ligand binding), second messengers (e.g., cAMP, cGMP, IP_3, DAG, or Ca^{2+}), ion flux, phosphorylation levels, transcription levels, neurotransmitter levels, and the like. In addition, the effects of binding on phenotypes associated with cancer can be measured, such assays include cell growth on soft agar; anchorage dependence; contact inhibition and density limitation of growth; cellular proliferation; cellular transformation; growth factor or serum dependence; tumor specific marker levels or tumor growth in nude mice. In addition, assays that measure metastatic phenotypes includes assays for invasiveness into Matrigel and other synthetic or natural matrices; metastasis *in vivo*; mRNA and protein expression in cells undergoing metastasis, and other characteristics of metastatic cancer cells. Other assays include assays for cell migration as described, for example, in Koshiba *et al. Clin Cancer Res.* 6:3530-5 (2000), or assays for expression of matrix degrading enzymes as described, for example, in Paoletti *Int J Oncol*; 18:11-6 (2001). "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities.

By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of a receptor protein sequence, e.g., functional, physical and chemical effects. Such functional effects can be measured by many means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties for the protein, measuring inducible

markers or transcriptional activation of the receptor protein; measuring binding activity or binding assays, e.g. binding to antibodies or other ligands, and measuring cellular proliferation.

The receptor or ligand proteins, antibodies, nucleic acids, modified proteins and cells containing such sequences can also be assayed by evaluating the effect of drug candidates on a "gene expression profile" or expression profile of polypeptides. In a preferred embodiment, the expression profiles are in conjunction with high throughput screening techniques to allow monitoring for expression profile genes after treatment with a candidate agent (e.g., Zlokarnik, *et al.*, *Science* 279:84-8 (1998); Heid, *Genome Res* 6:986-94, 1996).

The term "test compound" or "drug candidate" or "modulator" or grammatical equivalents as used herein describes any molecule, e.g., protein, oligopeptide, small organic molecule, polysaccharide, polynucleotide, etc., to be tested for the capacity to directly or indirectly alter the cancer phenotype or the expression of a cancer-associated sequence, e.g., a nucleic acid or protein sequence. In preferred embodiments, modulators alter receptor expression or activity, as measured in well known assays.

In certain embodiments, combinatorial libraries of potential modulators will be screened for an ability to bind to a receptor polypeptide or to modulate its activity. Conventionally, new chemical entities with useful properties are generated by identifying a chemical compound (called a "lead compound") with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

In one preferred embodiment, high throughput screening methods involve providing a library containing a large number of potential therapeutic compounds (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more assays to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library, such as a polypeptide (e.g., mutein) library, is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound

length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks (Gallop *et al., J. Med. Chem.* 37(9):1233-1251 (1994)).

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.,* U.S. Patent No. 5,010,175, Furka, *Pept. Prot. Res.* 37:487-493 (1991), Houghton *et al.*, *Nature*, 354:84-88 (1991)), peptoids (PCT Publication No WO 91/19735), encoded peptides (PCT Publication WO 93/20242), random bio-oligomers (PCT Publication WO 92/00091), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al., Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al., J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with a Beta-D-Glucose scaffolding (Hirschmann *et al., J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al., J. Amer. Chem.* Soc. 116:2661 (1994)), oligocarbamates (Cho, *et al., Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al., J. Org. Chem.* 59:658 (1994)). *See, generally,* Gordon *et al., J. Med. Chem.* 37:1385 (1994), nucleic acid libraries (*see, e.g.,* Strategene, Corp.), peptide nucleic acid libraries (*see, e.g.,* U.S. Patent 5,539,083), antibody libraries (*see, e.g.,* Vaughn *et al., Nature Biotechnology* 14(3):309-314 (1996), and PCT/US96/10287), carbohydrate libraries (*see, e.g.,* Liang *et al., Science* 274:1520-1522 (1996), and U.S. Patent No. 5,593,853), and small organic molecule libraries (*see, e.g.,* benzodiazepines, Baum, C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent No. 5,569,588; thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; pyrrolidines, U.S. Patent Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent No. 5,506,337; benzodiazepines, U.S. Patent No. 5,288,514; and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.,* 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.), which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use

with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.,* ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, *etc.*).

The assays to identify modulators are amenable to high throughput screening. Preferred assays include, for example, detection of enhancement or inhibition of binding of chemokines to the receptor protein. High throughput assays for the presence, absence, quantification, or other properties of particular nucleic acids or protein products are well known to those of skill in the art. Similarly, binding assays and reporter gene assays are similarly well known. Thus, for example, U.S. Patent No. 5,559,410 discloses high throughput screening methods for proteins, U.S. Patent No. 5,585,639 discloses high throughput screening methods for nucleic acid binding (i.e., in arrays), while U.S. Patent Nos. 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

In addition, high throughput screening systems are commercially available (*see, e.g.,* Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures, including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols for various high throughput systems. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

In one embodiment, modulators are proteins, often naturally occurring proteins or fragments of naturally occurring proteins. Thus, *e.g.*, cellular extracts containing proteins, or random or directed digests of proteinaceous cellular extracts, may be used. In this way libraries of proteins may be made for screening in the methods of the invention. Particularly preferred in this embodiment are libraries of bacterial, fungal, viral, and mammalian proteins, with the latter being preferred, and human proteins being especially preferred. Particularly useful test compound will be directed to the class of proteins to which the target belongs, *e.g.*, substrates for enzymes or ligands and receptors.

In a preferred embodiment, modulators are peptides of from about 5 to about 30 amino acids, with from about 5 to about 20 amino acids being preferred, and from about 7 to about 15 being particularly preferred. The peptides may be digests of naturally occurring proteins as is outlined above, random peptides, or "biased" random peptides. By "randomized" or grammatical equivalents herein is meant that each nucleic acid and peptide consists of essentially random nucleotides and amino acids, respectively. Since generally these random peptides (or nucleic acids, discussed below) are chemically synthesized, they may incorporate any nucleotide or amino acid at any position. The synthetic process can be designed to generate randomized proteins or nucleic acids, to allow the formation of all or most of the possible combinations over the length of the sequence, thus forming a library of randomized candidate bioactive proteinaceous agents.

In one embodiment, the library is fully randomized, with no sequence preferences or constants at any position. In a preferred embodiment, the library is biased. That is, some positions within the sequence are either held constant, or are selected from a limited number of possibilities. For example, in a preferred embodiment, the nucleotides or amino acid residues are randomized within a defined class, for example, of hydrophobic amino acids, hydrophilic residues, sterically biased (either small or large) residues, towards the creation of nucleic acid binding domains, the creation of cysteines, for cross-linking, prolines for SH-3 domains, serines, threonines, tyrosines or histidines for phosphorylation sites, etc., or to purines, etc.

As described above generally for proteins, nucleic acid modulating agents may be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. For example, digests of prokaryotic or eukaryotic genomes may be used as is outlined above for proteins.

In a preferred embodiment, the candidate compounds are organic chemical moieties, a wide variety of which are available in the literature.

After the candidate agent has been added and the cells allowed to incubate for some period of time, the sample containing a target sequence to be analyzed is added to the biochip. If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, electroporation, etc., with purification and/or amplification such as PCR performed as appropriate. For example, an *in vitro* transcription with labels covalently attached to the nucleotides is performed. Generally, the nucleic acids are labeled with biotin-FITC or PE, or with cy3 or cy5.

In a preferred embodiment, the target sequence is labeled with, for example, a fluorescent, a chemiluminescent, a chemical, or a radioactive signal, to provide a means of detecting the target sequence's specific binding to a probe. The label also can be an enzyme, such as, alkaline phosphatase or horseradish peroxidase, which when provided with an appropriate substrate produces a product that can be detected. Alternatively, the label can be a labeled compound or small molecule, such as an enzyme inhibitor, that binds but is not catalyzed or altered by the enzyme. The label also can be a moiety or compound, such as, an epitope tag or biotin which specifically binds to streptavidin. For the example of biotin, the streptavidin is labeled as described above, thereby, providing a detectable signal for the bound target sequence. Unbound labeled streptavidin is typically removed prior to analysis.

As will be appreciated by those in the art, these assays can be direct hybridization assays or can comprise "sandwich assays", which include the use of multiple probes, as is generally outlined in U.S. Patent Nos. 5,681,702, 5,597,909, 5,545,730, 5,594,117, 5,591,584, 5,571,670, 5,580,731, 5,571,670, 5,591,584, 5,624,802, 5,635,352, 5,594,118, 5,359,100, 5,124,246 and 5,681,697, all of which are hereby incorporated by reference. In this embodiment, in general, the target nucleic acid is prepared as outlined above, and then added to the biochip comprising a plurality of nucleic acid probes, under conditions that allow the formation of a hybridization complex.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions as outlined above. The assays are generally run under stringency conditions which allows formation of the label probe hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions to reduce non-specific binding.

The reactions outlined herein may be accomplished in a variety of ways. Components of the reaction may be added simultaneously, or sequentially, in different orders, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents. These include salts, buffers, neutral proteins, *e.g.* albumin, detergents, *etc.* which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Reagents that otherwise improve the efficiency of the

assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, *etc.*, may also be used as appropriate, depending on the sample preparation methods and purity of the target.

The assay data are analyzed to determine the expression levels, and changes in expression levels as between states, of individual genes, forming a gene expression profile.

Screens are performed to identify modulators of a cancer phenotype. In one embodiment, screening is performed to identify modulators that can induce or suppress a particular expression profile, thus preferably generating the associated phenotype. In another embodiment, *e.g.*, for diagnostic applications, having identified differentially expressed genes important in a particular state, screens can be performed to identify modulators that alter expression of individual genes. In an another embodiment, screening is performed to identify modulators that alter a biological function of the expression product of a differentially expressed gene. Again, having identified the importance of a gene in a particular state, screens are performed to identify agents that bind and/or modulate the biological activity of the gene product.

In addition screens can be done for genes that are induced in response to a candidate agent. After identifying a modulator based upon its ability to suppress an cancer expression pattern leading to a normal expression pattern, or to modulate a single cancer gene expression profile so as to mimic the expression of the gene from normal tissue, a screen as described above can be performed to identify genes that are specifically modulated in response to the agent. Comparing expression profiles between normal tissue and agent treated cancer tissue reveals genes that are not expressed in normal tissue or cancer tissue, but are expressed in agent treated tissue. These agent-specific sequences can be identified and used by methods described herein for cancer genes or proteins. In particular these sequences and the proteins they encode find use in marking or identifying agent treated cells. In addition, antibodies can be raised against the agent induced proteins and used to target novel therapeutics to the treated cancer tissue sample.

Thus, in one embodiment, a test compound is administered to a population of cancer cells, that have an associated cancer expression profile. By "administration" or "contacting" herein is meant that the candidate agent is added to the cells in such a manner as to allow the agent to act upon the cell, whether by uptake and intracellular action, or by action at the cell surface. In some embodiments, nucleic acid encoding a proteinaceous candidate agent (i.e., a peptide) may be put into a viral construct such as an adenoviral or retroviral construct, and added to the cell, such that expression of the peptide agent is accomplished, e.g., PCT US97/01019. Regulatable gene therapy systems can also be used.

Once the test compound has been administered to the cells, the cells can be washed if desired and are allowed to incubate under preferably physiological conditions for some period of time. The cells are then harvested and a new gene expression profile is generated, as outlined herein.

Thus, for example, cancer tissue may be screened for agents that modulate, e.g., induce or suppress the cancer phenotype. A change in at least one gene, preferably many, of the expression profile indicates that the agent has an effect on receptor activity. By defining such a signature for the cancer phenotype, screens for new drugs that alter the phenotype can be devised. With this approach, the drug target need not be known and need not be represented in the original expression screening platform, nor does the level of transcript for the target protein need to change.

Assays to identify compounds with modulating activity can be performed *in vitro*. For example, a receptor polypeptide is first contacted with a potential modulator and incubated for a suitable amount of time, e.g., from 0.5 to 48 hours. In one embodiment, the receptor polypeptide levels are determined *in vitro* by measuring the level of protein or mRNA. The level of protein is measured using immunoassays such as western blotting, ELISA and the like with an antibody that selectively binds to the receptor polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization, RNAse protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

Alternatively, a reporter gene system can be devised using the receptor protein promoter operably linked to a reporter gene such as luciferase, green fluorescent protein, CAT, or β-gal. The reporter construct is typically transfected into a cell. After treatment with a potential modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

In a preferred embodiment, binding assays are done. In general, purified or isolated gene product is used; that is, the gene products of one or more differentially expressed nucleic acids are made. For example, antibodies are generated to the protein gene products, and standard immunoassays are run to determine the amount of protein present. Alternatively, cells comprising the receptor proteins can be used in the assays.

Thus, in a preferred embodiment, the methods comprise combining receptor protein, e.g., CXCR4, and a candidate compound, and determining the binding of the

compound to the protein. Preferred embodiments utilize the human CXCR4 protein, although other mammalian proteins may also be used, for example for the development of animal models of human disease. In some embodiments, as outlined herein, variant or derivative CXCR4 proteins may be used.

Generally, in a preferred embodiment of the methods herein, the receptor protein or the candidate agent is non-diffusably bound to an insoluble support having isolated sample receiving areas (e.g. a microtiter plate, an array, etc.). The insoluble supports may be made of any composition to which the compositions can be bound, is readily separated from soluble material, and is otherwise compatible with the overall method of screening. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports include microtiter plates, arrays, membranes and beads. These are typically made of glass, plastic (e.g., polystyrene), polysaccharides, nylon or nitrocellulose, teflon™, etc. Microtiter plates and arrays are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples. The particular manner of binding of the composition is not crucial so long as it is compatible with the reagents and overall methods of the invention, maintains the activity of the composition and is nondiffusable. Preferred methods of binding include the use of antibodies (which do not sterically block either the ligand binding site or activation sequence when the protein is bound to the support), direct binding to "sticky" or ionic supports, chemical crosslinking, the synthesis of the protein or agent on the surface, etc. Following binding of the protein or agent, excess unbound material is removed by washing. The sample receiving areas may then be blocked through incubation with bovine serum albumin (BSA), casein or other innocuous protein or other moiety.

In a preferred embodiment, the receptor protein is bound to the support, and a test compound is added to the assay. Alternatively, the candidate agent is bound to the support and the receptor protein is added. Novel binding agents include specific antibodies, non-natural binding agents identified in screens of chemical libraries, peptide analogs, etc. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, functional assays (phosphorylation assays, etc.) and the like.

The determination of the binding of the test modulating compound to the receptor protein may be done in a number of ways. In a preferred embodiment, the compound is labeled, and binding determined directly, *e.g.*, by attaching all or a portion of

the receptor protein to a solid support, adding a labeled candidate agent (e.g., a fluorescent label), washing off excess reagent, and determining whether the label is present on the solid support. Various blocking and washing steps may be utilized as appropriate.

In some embodiments, only one of the components is labeled, *e.g.*, the proteins (or proteinaceous candidate compounds) can be labeled. Alternatively, more than one component can be labeled with different labels, e.g., ^{125}I for the proteins and a fluorophor for the compound. Proximity reagents, e.g., quenching or energy transfer reagents are also useful.

In one embodiment, the binding of the test compound is determined by competitive binding assay. The competitor is a binding moiety known to bind to the target molecule (e.g., a CXCR4 protein), such as an antibody, peptide, binding partner, ligand, etc. Under certain circumstances, there may be competitive binding between the compound and the binding moiety, with the binding moiety displacing the compound. In one embodiment, the test compound is labeled. Either the compound, or the competitor, or both, is added first to the protein for a time sufficient to allow binding, if present. Incubations may be performed at a temperature which facilitates optimal activity, typically between 4 and 40°C. Incubation periods are typically optimized, *e.g.*, to facilitate rapid high throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

In a preferred embodiment, the competitor is added first, followed by the test compound. Displacement of the competitor is an indication that the test compound is binding to the receptor protein and thus is capable of binding to, and potentially modulating, the activity of the receptor protein. In this embodiment, either component can be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the test compound is labeled, the presence of the label on the support indicates displacement.

In an alternative embodiment, the test compound is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate that the test compound is bound to the receptor protein with a higher affinity. Thus, if the test compound is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate that the test compound is capable of binding to the receptor protein.

In a preferred embodiment, the methods comprise differential screening to identity agents that are capable of modulating the activity of the receptor proteins. In this embodiment, the methods comprise combining a receptor protein and a competitor in a first sample. A second sample comprises a test compound, a receptor protein, and a competitor. The binding of the competitor is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of an agent capable of binding to the receptor protein and potentially modulating its activity. That is, if the binding of the competitor is different in the second sample relative to the first sample, the agent is capable of binding to the receptor protein.

Alternatively, differential screening is used to identify drug candidates that bind to the native receptor protein, but cannot bind to modified receptor proteins. The structure of the CXCR4 protein may be modeled, and used in rational drug design to synthesize agents that interact with that site. Drug candidates that affect the activity of a receptor protein are also identified by screening drugs for the ability to either enhance or reduce the activity of the protein.

Positive controls and negative controls may be used in the assays. Preferably control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in an order that provides for the requisite binding.

In a preferred embodiment, the invention provides methods for screening for a compound capable of modulating the activity of a receptor protein. The methods comprise adding a test compound, as defined above, to a cell comprising receptor proteins. Preferred cell types include almost any cell. The cells contain a recombinant nucleic acid that encodes a receptor protein. In a preferred embodiment, a library of candidate agents are tested on a plurality of cells.

In one aspect, the assays are evaluated in the presence or absence or previous or subsequent exposure of physiological signals, for example hormones, antibodies, peptides, antigens, cytokines, growth factors, action potentials, pharmacological agents including chemotherapeutics, radiation, carcinogenics, or other cells (i.e. cell-cell contacts). In another example, the determinations are determined at different stages of the cell cycle process.

In this way, compounds that modulate receptor agents are identified. Compounds with pharmacological activity are able to enhance or interfere with the activity of the receptor protein. Once identified, similar structures are evaluated to identify critical structural features of the compound.

In one embodiment, a method of inhibiting division of a cell expressing a receptor is provided. The method comprises administration of a receptor inhibitor. In another embodiment, a method of inhibiting CXCR4 is provided. The method comprises administration of a CXCR4 inhibitor. In a further embodiment, methods of treating cells or individuals with cancer are provided. The method comprises administration of a CXCR4 inhibitor.

In one embodiment, a CXCR4 inhibitor is an antibody as discussed above. In another embodiment, the CXCR4 inhibitor is an antisense molecule.

A variety of cell growth, proliferation, and metastasis assays are known to those of skill in the art, as described below.

Soft agar growth or colony formation in suspension

Normal cells require a solid substrate to attach and grow. When the cells are transformed, they lose this phenotype and grow detached from the substrate. For example, transformed cells can grow in stirred suspension culture or suspended in semi-solid media, such as semi-solid or soft agar. The transformed cells, when transfected with tumor suppressor genes, regenerate normal phenotype and require a solid substrate to attach and grow. Soft agar growth or colony formation in suspension assays can be used to identify modulators of receptor sequences, which when expressed in host cells, inhibit abnormal cellular proliferation and transformation. A therapeutic compound would reduce or eliminate the host cells' ability to grow in stirred suspension culture or suspended in semi-solid media, such as semi-solid or soft.

Techniques for soft agar growth or colony formation in suspension assays are described in Freshney, *Culture of Animal Cells a Manual of Basic Technique* (3rd ed., 1994),

herein incorporated by reference. *See also*, the methods section of Garkavtsev *et al.* (1996), *supra*, herein incorporated by reference.

Contact inhibition and density limitation of growth

Normal cells typically grow in a flat and organized pattern in a petri dish until they touch other cells. When the cells touch one another, they are contact inhibited and stop growing. When cells are transformed, however, the cells are not contact inhibited and continue to grow to high densities in disorganized foci. Thus, the transformed cells grow to a higher saturation density than normal cells. This can be detected morphologically by the formation of a disoriented monolayer of cells or rounded cells in foci within the regular pattern of normal surrounding cells. Alternatively, labeling index with (^{3}H)-thymidine at saturation density can be used to measure density limitation of growth. *See* Freshney (1994), *supra*. The transformed cells, when transfected with tumor suppressor genes, regenerate a normal phenotype and become contact inhibited and would grow to a lower density.

In this assay, labeling index with (^{3}H)-thymidine at saturation density is a preferred method of measuring density limitation of growth. Transformed host cells are transfected with a receptor sequence and are grown for 24 hours at saturation density in non-limiting medium conditions. The percentage of cells labeling with (^{3}H)-thymidine is determined autoradiographically. *See*, Freshney (1994), *supra*.

Growth factor or serum dependence

Transformed cells have a lower serum dependence than their normal counterparts (*see, e.g.*, Temin, *J. Natl. Cancer Inst.* 37:167-175 (1966); Eagle *et al.*, *J. Exp. Med.* 131:836-879 (1970)); Freshney, *supra*. This is in part due to release of various growth factors by the transformed cells. Growth factor or serum dependence of transformed host cells can be compared with that of control.

Tumor specific markers levels

Tumor cells release an increased amount of certain factors (hereinafter "tumor specific markers") than their normal counterparts. For example, plasminogen activator (PA) is released from human glioma at a higher level than from normal brain cells (*see, e.g.*, Gullino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth.* in *Biological Responses in Cancer*, pp. 178-184 (Mihich (ed.) 1985)). Similarly, Tumor

angiogenesis factor (TAF) is released at a higher level in tumor cells than their normal counterparts. *See, e.g.,* Folkman, *Angiogenesis and Cancer, Sem. Cancer Biol.* (1992)).

Various techniques which measure the release of these factors are described in Freshney (1994), *supra*. Also, *see*, Unkless *et al.* , *J. Biol. Chem.* 249:4295-4305 (1974); Strickland & Beers, *J. Biol. Chem.* 251:5694-5702 (1976); Whur *et al.*, *Br. J. Cancer* 42:305-312 (1980); Gullino, *Angiogenesis, tumor vascularization, and potential interference with tumor growth.* in *Biological Responses in Cancer*, pp. 178-184 (Mihich (ed.) 1985); Freshney *Anticancer Res.* 5:111-130 (1985).

Invasiveness into Matrigel

The degree of invasiveness into Matrigel or some other extracellular matrix constituent can be used as an assay to identify compounds that modulate receptor sequences. Tumor cells exhibit a good correlation between malignancy and invasiveness of cells into Matrigel or some other extracellular matrix constituent. In this assay, tumorigenic cells are typically used as host cells. Expression of a tumor suppressor gene in these host cells would decrease invasiveness of the host cells.

Techniques described in Freshney (1994), *supra*, can be used. Briefly, the level of invasion of host cells can be measured by using filters coated with Matrigel or some other extracellular matrix constituent. Penetration into the gel, or through to the distal side of the filter, is rated as invasiveness, and rated histologically by number of cells and distance moved, or by prelabeling the cells with ^{125}I and counting the radioactivity on the distal side of the filter or bottom of the dish. *See, e.g.,* Freshney (1984), *supra*.

Tumor growth in vivo

Effects of CXCR4 sequences on cell growth can be tested in transgenic or immune-suppressed mice. Knock-out transgenic mice can be made, in which the receptor gene is disrupted or in which a receptor gene is inserted. Knock-out transgenic mice can be made by insertion of a marker gene or other heterologous gene into the endogenous receptor gene site in the mouse genome via homologous recombination. Such mice can also be made by substituting the endogenous receptor gene with a mutated version of the receptor gene, or by mutating the endogenous receptor gene, e.g., by exposure to carcinogens.

A DNA construct is introduced into the nuclei of embryonic stem cells. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is re-implanted into a recipient female. Some of these embryos develop into chimeric mice

that possess germ cells partially derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (*see, e.g.,* Capecchi *et al., Science* 244:1288 (1989)). Chimeric targeted mice can be derived according to Hogan *et al., Manipulating the Mouse Embryo*: *A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed., IRL Press, Washington, D.C., (1987).

Alternatively, various immune-suppressed or immune-deficient host animals can be used. For example, genetically athymic "nude" mouse (*see, e.g.,* Giovanella *et al., J. Natl. Cancer Inst.* 52:921 (1974)), a SCID mouse, a thymectomized mouse, or an irradiated mouse (*see, e.g.,* Bradley *et al., Br. J. Cancer* 38:263 (1978); Selby *et al., Br. J. Cancer* 41:52 (1980)) can be used as a host. Transplantable tumor cells (typically about 10^6 cells) injected into isogenic hosts will produce invasive tumors in a high proportions of cases, while normal cells of similar origin will not. In hosts which developed invasive tumors, cells expressing CXCR4 sequences are injected subcutaneously. After a suitable length of time, preferably 4-8 weeks, tumor growth is measured (*e.g.*, by volume or by its two largest dimensions) and compared to the control. Tumors that have statistically significant reduction (using, *e.g.*, Student's T test) are said to have inhibited growth.

Rheumatoid Arthritis

A number of animal models of this disease are known. The immune response in mice to native type II collagen has been used to establish an experimental model for arthritis with a number of histological and pathological features resembling human RA. Susceptibility to collagen-induced arthritis (CIA) in mice has been mapped to the H-2 I region, particularly the I-A subregion. Huse, *et al. ., Fed. Proc.* 43:1820 (1984).

Mice from a susceptible strain, DBA-1 are caused to have CIA by treatment of the mice with native type II collagen, using the technique described in Wooley and Luthra, J. *Immunol.* 134:2366 (1985), incorporated herein by reference.

In another model, adjuvant arthritis in rats is an experimental model for human arthritis, and a prototype of autoimmune arthritis triggered by bacterial antigens, Holoschitz, et al., *Prospects of Immunology* (CRC Press) (1986); Pearson *Arthritis Rheum.* 7:80 (1964). The disease the result of a cell-mediated immune response, as evidenced by its transmissibility by a clone of T cells which were reactive against the adjuvant (MT); the target self-antigen in the disease, based upon studies with the same cloned cells, appears to be part(s) of a proteoglycan molecule of cartilage.

Adjuvant disease in rats is produced by a single injection of Freund's adjuvant (killed tubercle bacilli or chemical fractions of it, mineral oil, and an emulsifying agent) given into several depot sites, preferably intracutaneously or into a paw or the base of the tail. The adjuvant is given in the absence of other antigens.

The effect of complex treatment of manifestations of the disease are monitored. These manifestations are histopathological, and include an acute and subacute synovitis with proliferation of synovial lining cells, predominantly a mononuclear infiltration of the articular and particular tissues, the invasion of bone and articular cartilage by connective tissue pannus, and periosteal new bone formation, especially adjacent to affected joints. In severe or chronic cases, destructive changes occur, as do fibrous or bony ankylosis. These histopathological symptoms are expected to appear in control animals at about 12 days after sensitization to the Freund's adjuvant.

Polynucleotide modulators of disease

Antisense Polynucleotides

In certain embodiments, the activity of a receptor protein is downregulated, or entirely inhibited, by the use of antisense polynucleotide, *i.e.*, a nucleic acid complementary to, and which can preferably hybridize specifically to, a coding mRNA nucleic acid sequence, *e.g.*, a receptor protein mRNA, or a subsequence thereof. Binding of the antisense polynucleotide to the mRNA reduces the translation and/or stability of the mRNA.

In the context of this invention, antisense polynucleotides can comprise naturally-occurring nucleotides, or synthetic species formed from naturally-occurring subunits or their close homologs. Antisense polynucleotides may also have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur containing species which are known for use in the art. Analogs are comprehended by this invention so long as they function effectively to hybridize with the receptor protein mRNA. *See, e.g.,* Isis Pharmaceuticals, Carlsbad, CA; Sequitor, Inc., Natick, MA.

Such antisense polynucleotides can readily be synthesized using recombinant means, or can be synthesized *in vitro*. Equipment for such synthesis is sold by several vendors, including Applied Biosystems. The preparation of other oligonucleotides such as phosphorothioates and alkylated derivatives is also well known to those of skill in the art.

Antisense molecules as used herein include antisense or sense oligonucleotides. Sense oligonucleotides can, e.g., be employed to block transcription by binding to the anti-sense strand. The antisense and sense oligonucleotide comprise a single-

stranded nucleic acid sequence (either RNA or DNA) capable of binding to target mRNA (sense) or DNA (antisense) sequences for receptor molecules. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment generally at least about 14 nucleotides, preferably from about 14 to 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein & Cohen *Cancer Res.* 48:2659 (1988) and van der Krol *et al.*(*BioTechniques* 6:958 (1988).

Ribozymes

In addition to antisense polynucleotides, ribozymes can be used to target and inhibit transcription of receptor nucleotide sequences. A ribozyme is an RNA molecule that catalytically cleaves other RNA molecules. Different kinds of ribozymes have been described, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNase P, and axhead ribozymes (*see, e.g.,* Castanotto *et al.*, *Adv. in Pharmacology* 25: 289-317 (1994) for a general review of the properties of different ribozymes).

The general features of hairpin ribozymes are described, *e.g.*, in Hampel *et al.*, *Nucl. Acids Res.* 18:299-304 (1990); European Patent Publication No. 0 360 257; U.S. Patent No. 5,254,678. Methods of preparing are well known to those of skill in the art (*see, e.g.,* WO 94/26877; Ojwang *et al.*, *Proc. Natl. Acad. Sci.* USA 90:6340-6344 (1993); Yamada *et al.*, *Human Gene Therapy* 1:39-45 (1994); Leavitt *et al.*, *Proc. Natl. Acad. Sci. USA* 92:699-703 (1995); Leavitt *et al.*, *Human Gene Therapy* 5:1151-120 (1994); and Yamada *et al.*, *Virology* 205: 121-126 (1994)).

Polynucleotide modulators of receptor may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell. Alternatively, a polynucleotide modulator of receptor may be introduced into a cell containing the target nucleic acid sequence, e.g., by formation of an polynucleotide-lipid complex, as described in WO 90/10448. It is understood that the use of antisense molecules or knock out and knock in models may also be used in screening assays as discussed above, in addition to methods of treatment.

Thus, in one embodiment, methods of modulating receptor in cells or organisms are provided. In one embodiment, the methods comprise administering to a cell an anti-receptor antibody that reduces or eliminates the biological activity of an endogenous receptor protein.

In one embodiment, the receptor proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to those receptor proteins. Similarly, the receptor proteins can be coupled, using standard technology, to affinity chromatography columns. These columns may then be used to purify receptor antibodies useful for production, diagnostic, or therapeutic purposes. In a preferred embodiment, the antibodies are generated to epitopes unique to a specific receptor protein; that is, the antibodies show little or no cross-reactivity to other proteins. The receptor antibodies may be coupled to standard affinity chromatography columns and used to purify receptor proteins. The antibodies may also be used as blocking polypeptides, as outlined above, since they will specifically bind to the receptor protein.

Administration of pharmaceutical and vaccine compositions

In one embodiment, a therapeutically effective dose of a receptor or ligand protein or modulator thereof, is administered to a patient for treatment of cancers, such as ovarian, lung, colorectal, bladder, head and neck, renal, stomach, uterine, acute lymphoblastic leukemia, cervical, glioblastoma, prostate, or breast cancer or the prevention of angiogenesis. As noted above, the CXCR4 compositions can be used to treat rheumatoid arthritis as well. Rheumatoid arthritis (RA) is a chronic inflammatory disease resulting from an immune response to proteins found in the synovial fluid.

By "therapeutically effective dose" herein is meant a dose that produces effects for which it is administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (e.g., Ansel *et al.*, *Pharmaceutical Dosage Forms and Drug Delivery*; Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992), Dekker, ISBN 0824770846, 082476918X, 0824712692, 0824716981; Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)). As is known in the art, adjustments for protein degradation, systemic versus localized delivery, and rate of new protease synthesis, as well as the age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals. Thus the methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, preferably a primate, and in the most preferred embodiment the patient is human.

The administration of the receptor proteins and modulators thereof of the present invention can be done in a variety of ways as discussed above, including, but not limited to, orally, subcutaneously, intravenously, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary, vaginally, rectally, or intraocularly.

The activity of the receptors can be specifically inhibited by many methods known to those of skill in the art, such as small molecule antagonists, peptide antagonists, receptor-specific inhibition of signaling, ligand variants and homologs, and transcriptional control of receptor expression levels.

The most widely known CXCR4 and other chemokine receptor antagonists are bicyclams, a family of chemical compounds that are composed of two cyclam (1,4,8,11-tetraazacyclo-tetradecane) units linked by an aliphatic or aromatic linker (Donzella *et al. Nat Med* 4:72-77 (1998); Schols *et al. Antiviral Res* 35:147-156(1997)). Numerous derivatives of the prototypic bicyclam, AMD3100, have been synthesized to optimize the CXCR4 antagonist activity of these compounds (Bridger *et al. J Med Chem* 42:3971-81 (1999)).

Truncated peptide analogues of polyphemusin, which is an antimicrobial peptide found in the hemocytes of American horseshoe crabs, have also been identified as specific CXCR4 antagonists. Like bicyclams, these compounds are low molecular weight compounds. However, they have a different structure and are active against bicyclam-resistant HIV strains. (Tamamura *et al. Biochem Biophys Res Commun 253:877-82* (1998); Xu *et al. AIDS Res Hum Retroviruses* 15:419-27 (1999); Tamamura *et al. Bioorg Med Chem Lett* 10 2633-7 (2000); Tamamura *et al. Bioorg Med Chem Lett* 11:359-362 (2001)). Related molecules will be useful antagonists or agonists of the other receptors.

Other antagonists of CXCR4 are ALX40-4C, a nonapeptide solely existing of arginine residues (Doranz *et al. J Exp Med* 186:1395-400 (1997)); the polycationic peptoid CGP64222 (Hamy *et al. Proc Natl. Acad Sci USA* 94: 3548-3553 (1997); Daelemans *et al. Mol Pharmacol* 57:116-124 (2000)), and compounds described in Naya *et al.* (U.S. Patent No. 6,140,338) and Atsma *et al.* (WO0056729).

Peptide antagonists derived from naturally occurring CXCR4 ligands are also known. Certain analogues of N-terminal peptides of the chemokine SDF-1 (stromal cell-derived factor-1) are CXCR4 antagonists (Loetscher *et al. J Biol Chem* 273:22279-22283

(1998)). In addition, Staudinger *et al.* have demonstrated that another CXCR4 ligand, the HIV-1 envelope protein gp120, effectively antagonizes the effect of SDF-1 (*Biochem Biophys Res Commun.* 280:1003-1007). Ligand variants or homologs may serve as antagonists or agonists.

CXCR4 activity can also be modulated by interfering with the receptor itself, rather than inhibiting agonist binding. Tarasova *et al.* demonstrate expression of a peptide derived from the transmembrane domains of CXCR4 inhibits receptor signaling and HIV replication at concentrations as low as 0.2 uM (*J Biol Chem* 274:34911-34915 (1999)). Another method to reduce receptor activity is to lower expression levels using many methods known to those of skill in the art, such as antisense oligonucleotides that hybridize specifically with chromosomal DNA and/or RNA (Takeshi *et al.* EP1050583) and liposomal compositions of vectors encoding hammerhead ribozymal DNA sequences directed towards CXCR4 mRNA (Eagles *et al.* WO/9936518). Similar strategies should be applicable to other receptors.

The pharmaceutical compositions of the present invention comprise a receptor protein or modulator in a form suitable for administration to a patient. In the preferred embodiment, the pharmaceutical compositions are in a water soluble form, such as being present as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. "Pharmaceutically acceptable acid addition salt" refers to those salts that retain the biological effectiveness of the free bases and that are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. "Pharmaceutically acceptable base addition salts" include those derived from inorganic bases such as sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Particularly preferred are the ammonium, potassium, sodium, calcium, and magnesium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, and ethanolamine.

The pharmaceutical compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; fillers such as microcrystalline cellulose, lactose, corn and other starches; binding agents; sweeteners and other flavoring agents; coloring agents; and polyethylene glycol.

The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include, but are not limited to, powder, tablets, pills, capsules and lozenges. It is recognized that receptor protein modulators (e.g., antibodies, antisense constructs, ribozymes, small organic molecules, *etc.*) when administered orally, should be protected from digestion. This is typically accomplished either by complexing the molecule(s) with a composition to render it resistant to acidic and enzymatic hydrolysis, or by packaging the molecule(s) in an appropriately resistant carrier, such as a liposome or a protection barrier. Means of protecting agents from digestion are well known in the art.

The compositions for administration will commonly comprise a receptor protein modulator dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of active agent in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs (e.g., *Remington's Pharmaceutical Science* (15th ed., 1980) and Goodman & Gillman, *The Pharmacological Basis of Therapeutics* (Hardman *et al.*, eds., 1996)).

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Substantially higher dosages are possible in topical administration. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art, *e.g.*, *Remington's Pharmaceutical Science* and Goodman and Gillman, *The Pharmacological Basis of Therapeutics*, *supra*.

The compositions containing modulators of receptor proteins can be administered for therapeutic or prophylactic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease (e.g., a cancer) in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health. Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the agents of this invention to effectively treat the patient. An amount of modulator that is capable of preventing or slowing the development of cancer in a mammal is referred to as a "prophylactically effective dose." The particular dose required for a prophylactic treatment will depend upon the medical condition and history of the mammal, the particular cancer being prevented, as well as other factors such as age, weight, gender, administration route, efficiency, *etc.* Such prophylactic treatments may be used, *e.g.*, in a mammal who has previously had cancer to prevent a recurrence of the cancer, or in a mammal who is suspected of having a significant likelihood of developing cancer.

It will be appreciated that the present receptor protein-modulating compounds can be administered alone or in combination with additional modulating compounds or with other therapeutic agent, *e.g.*, other anti-cancer agents or treatments.

In numerous embodiments, one or more nucleic acids, *e.g.*, polynucleotides comprising nucleic acid sequences such as antisense polynucleotides or ribozymes, will be introduced into cells, *in vitro* or *in vivo*. The present invention provides methods, reagents, vectors, and cells useful for expression of receptor polypeptides and nucleic acids using *in vitro* (cell-free), *ex vivo* or *in vivo* (cell or organism-based) recombinant expression systems.

The particular procedure used to introduce the nucleic acids into a host cell for expression of a protein or nucleic acid is application specific. Many procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, spheroplasts, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.*, Berger & Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 (Berger), Ausubel *et al.*, eds., *Current Protocols* (supplemented through 1999), and Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual* (2nd ed., Vol. 1-3, 1989.

In a preferred embodiment, receptor proteins and modulators are administered as therapeutic agents, and can be formulated as outlined above. Similarly, receptor genes (including both the full-length sequence, partial sequences, or regulatory sequences of the receptor coding regions) can be administered in a gene therapy application. These receptor genes can include antisense applications, either as gene therapy (i.e. for incorporation into the genome) or as antisense compositions, as will be appreciated by those in the art.

Receptor polypeptides and polynucleotides can also be administered as vaccine compositions to stimulate HTL, CTL and antibody responses.. Such vaccine compositions can include, for example, lipidated peptides (*see, e.g.*, Vitiello, A. *et al.*, *J. Clin. Invest.* 95:341 (1995)), peptide compositions encapsulated in poly(DL-lactide-co-glycolide) ("PLG") microspheres (*see, e.g.,* Eldridge, *et al.*, *Molec. Immunol.* 28:287-294, (1991); Alonso *et al.*, *Vaccine* 12:299-306 (1994); Jones *et al.*, *Vaccine* 13:675-681 (1995)), peptide compositions contained in immune stimulating complexes (ISCOMS) (*see, e.g.,* Takahashi *et al.*, *Nature* 344:873-875 (1990); Hu *et al.*, *Clin Exp Immunol.* 113:235-243 (1998)), multiple antigen peptide systems (MAPs) (*see, e.g.*, Tam, *Proc. Natl. Acad. Sci. U.S.A.* 85:5409-5413 (1988); Tam, *J. Immunol. Methods* 196:17-32 (1996)), peptides formulated as multivalent peptides; peptides for use in ballistic delivery systems, typically crystallized peptides, viral delivery vectors (Perkus, *et al.*, In: *Concepts in vaccine development (*Kaufmann, ed., p. 379, 1996); Chakrabarti, *et al.*, *Nature* 320:535 (1986); Hu *et al.*, *Nature* 320:537 (1986); Kieny, *et al.*, *AIDS Bio/Technology* 4:790 (1986); Top *et al.*, *J. Infect. Dis.* 124:148 (1971); Chanda *et al.*, *Virology* 175:535 (1990)), particles of viral or synthetic origin (*see, e.g.*, Kofler *et al.*, *J. Immunol. Methods.* 192:25 (1996); Eldridge *et al.*, *Sem. Hematol.* 30:16 (1993); Falo *et al.*, *Nature Med.* 7:649 (1995)), adjuvants (Warren *et al.*, *Annu. Rev. Immunol.* 4:369 (1986); Gupta *et al.*, *Vaccine* 11:293 (1993)), liposomes (Reddy *et al.*, *J. Immunol.* 148:1585 (1992); Rock, *Immunol. Today* 17:131 (1996)), or, naked or particle absorbed cDNA (Ulmer, *et al.*, *Science* 259:1745 (1993); Robinson *et al.*, *Vaccine* 11:957 (1993); Shiver *et al.*, In: *Concepts in vaccine development* (Kaufmann, ed., p. 423, 1996); Cease & Berzofsky, *Annu. Rev. Immunol.* 12:923 (1994) and Eldridge *et al.*, *Sem. Hematol.* 30:16 (1993)). Toxin-targeted delivery technologies, also known as receptor mediated targeting, such as those of Avant Immunotherapeutics, Inc. (Needham, Massachusetts) may also be used.

Vaccine compositions often include adjuvants. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available

as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Vaccines can be administered as nucleic acid compositions wherein DNA or RNA encoding one or more of the polypeptides, or a fragment thereof, is administered to a patient. This approach is described, for instance, in Wolff *et. al.*, *Science* 247:1465 (1990) as well as U.S. Patent Nos. 5,580,859; 5,589,466; 5,804,566; 5,739,118; 5,736,524; 5,679,647; WO 98/04720; and in more detail below. Examples of DNA-based delivery technologies include "naked DNA", facilitated (bupivicaine, polymers, peptide-mediated) delivery, cationic lipid complexes, and particle-mediated ("gene gun") or pressure-mediated delivery (*see, e.g.,* U.S. Patent No. 5,922,687).

For therapeutic or prophylactic immunization purposes, the peptides of the invention can be expressed by viral or bacterial vectors. Examples of expression vectors include attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus, for example, as a vector to express nucleotide sequences that encode receptor polypeptides or polypeptide fragments. Upon introduction into a host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits an immune response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, U.S. Patent No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al.*, *Nature* 351:456-460 (1991). A wide variety of other vectors useful for therapeutic administration or immunization *e.g.* adeno and adeno-associated virus vectors, retroviral vectors, *Salmonella typhi* vectors, detoxified anthrax toxin vectors, and the like, will be apparent to those skilled in the art from the description herein (*see, e.g.,* Shata *et al.*, *Mol Med Today* 6:66-71 (2000); Shedlock *et al.*, *J Leukoc Biol* 68:793-806 (2000); Hipp *et al.*, *In Vivo* 14:571-85 (2000)).

Methods for the use of genes as DNA vaccines are well known, and include placing a receptor gene or portion of a receptor gene under the control of a regulatable promoter or a tissue-specific promoter for expression in a receptor targetted patient. The receptor gene used for DNA vaccines can encode full-length receptor proteins, but more

preferably encodes portions of the receptor proteins including peptides derived from the protein. In one embodiment, a patient is immunized with a DNA vaccine comprising a plurality of nucleotide sequences derived from a receptor gene. For example, receptor genes or sequence encoding subfragments of a receptor protein are introduced into expression vectors and tested for their immunogenicity in the context of Class I MHC and an ability to generate cytotoxic T cell responses. This procedure provides for production of cytotoxic T cell responses against cells which present antigen, including intracellular epitopes.

In a preferred embodiment, the DNA vaccines include a gene encoding an adjuvant molecule with the DNA vaccine. Such adjuvant molecules include cytokines that increase the immunogenic response to the receptor polypeptide encoded by the DNA vaccine. Additional or alternative adjuvants are available.

In another preferred embodiment receptor genes find use in generating animal models of cancer. Animal models of cancer find use in screening for receptor modulators. For example, transgenic animals can be generated that overexpress the receptor protein. Depending on the desired expression level, promoters of various strengths can be employed to express the transgene. Also, the number of copies of the integrated transgene can be determined and compared for a determination of the expression level of the transgene. Animals generated by such methods find use as animal models of cancer and are additionally useful in screening for modulators of receptor-related cancer.

Kits for Use in Diagnostic and/or Prognostic Applications

For use in diagnostic, research, and therapeutic applications suggested above, kits are also provided by the invention. In the diagnostic and research applications such kits may include any or all of the following: assay reagents, buffers, receptor nucleic acids or antibodies, hybridization probes and/or primers, antisense polynucleotides, ribozymes, dominant negative receptor polypeptides or polynucleotides, small molecule inhibitors of receptor, *etc.* A therapeutic product may include sterile saline or another pharmaceutically acceptable emulsion and suspension base.

In addition, the kits may include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the

like. Such media may include addresses to internet sites that provide such instructional materials.

The present invention also provides for kits for screening for modulators of particular receptors. Such kits can be prepared from readily available materials and reagents. For example, such kits directed to CXCR4 can comprise one or more of the following materials: a CXCR4 polypeptide or polynucleotide, reaction tubes, and instructions for testing CXCR4 activity. Optionally, the kit contains biologically active CXCR4 protein. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. Diagnosis would typically involve evaluation of a plurality of genes or products. The genes will be selected based on correlations with important parameters in disease which may be identified in historical or outcome data.

It is understood that the examples described above in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All publications, sequences of accession numbers, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.